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Cellulose synthesis genes *CESA6* and *CSI1* are important for salt stress tolerance in *Arabidopsis*

Soil salinity is a widespread abiotic stress constraint threatening agricultural production, as it severely inhibits growth and development of crops. Several salt stress signaling pathways have been discovered in the model plant *Arabidopsis thaliana*, which include components for sensing the stress, transmitting the signal and regulating the downstream genes (Deinlein et al. 2014; Julkowska and Testerink 2015). In nature, plant roots directly encounter heterogeneous soils and therefore their responses to saline environments require dynamic changes in growth, a process in which plant cell walls play important roles (Tenhaken 2015). However, the molecular mechanisms underlying cell wall remodeling under stress conditions are still unclear.

To identify salt stress tolerance determinants, an Ethyl methane sulfonate (EMS) screening was carried out in the *Arabidopsis bzip60* (Fujita et al. 2007) mutant background. One of the salt hyper-sensitive mutants, *she1*, grew relatively normally in the 1/2 MS medium; however, *she1* mutants were more sensitive to mild salt stress compared to the control (Figures 1A, S1A). In contrast to shoot growth, root growth of *she1* was more significantly inhibited by salt stress; swollen and larger roots were observed with salt-stressed *she1* plants (Figure S1B). To investigate whether the salt hypersensitive phenotype of *she1* is attributed to ion toxicity or osmotic stress, *she1* seedlings were treated with various concentrations of NaCl, KCl, LiCl and mannitol. It turned out that *she1* mutants were hyper-sensitive to both ionic and osmotic stresses in roots (Figure S2A-D).

SHE1 was isolated as a non-sense mutation in locus AT5G64740, leading to a conversion of R27 to a premature stop codon (Figure S3; Table S1). AT5G64740 encodes a well-known cellulose synthase CESA6 (Fagard et al. 2000). To confirm that *SHE1* is *CESA6*, two alleles of *cesa6* mutants, *prc1-1* and *ixr2-1* (Figure S3), were obtained and their sensitivities to salt stress were evaluated. It was found that *prc1-1* had a similar phenotype as she1 did (Figure 1A). The *irx2-1* mutant might not be a knock-out mutant since *irx2-1* has a mild hypocotyl elongation phenotype when growing in the dark (Desprez et al. 2002). To further confirm that *she1* is allelic to *prc1-1*, *she1* was crossed to *prc1-1*, and it was found that all successfully crossed F1 plants were salt hyper-sensitive (Figure S4A, B). The salt-sensitive phenotype of *she1* was not dependent on *bZIP60* mutation (Figure S4C). Under mild salt stress conditions, more lateral roots were observed in either *she1* or *she1* wild-type (wt) plants, which was related to auxin, since the auxin efflux inhibitor 1-N-naphthylphthalamic acid (NPA) dramatically inhibited lateral root emergence in the *she1* (wt) plants (Figure S5A,B). These results indicated that the cellulose synthase gene *CESA6*/*SHE1* is important for salt stress tolerance in *Arabidopsis*.

To understand why *she1* was hyper-sensitive to salt stress, roots were stained with various chemicals. When the wt and *cesa6* mutant plants were grown on normal medium, propidium iodide (PI) nicely outlined root cells in the elongation zone; when a low concentration of NaCl was present in the growth medium, PI staining was observed in both the cytoplasm and nucleus in root cells of *she1* (wt) and *prc1-1* mutants (Figure 1B), indicating that CESA6 mutation affects membrane integrity under salt stress conditions. The meristematic or elongation zone of roots was stained with fluorescein diacetate (FDA), which is an indicator of cell viability. The low concentration of NaCl in the growth medium did not affect the esterase activity in roots of the wt but it dramatically decreased the esterase activity in roots of the *she1* (wt) and *prc1-1* mutants, especially in the elongation zone (Figure 1C). To examine potential cell wall defects, roots of wt and *cesa6* mutants were stained with calcofluor (Fagard et al. 2000). Longitudinal sections under confocal microscopy showed that mild salt stress did not affect the signal intensity in the wt, but decreased the fluorescence in the elongation zone of *she1* (wt) and *prc1-1* mutants in the root vasculature regions (Figure 1D). These results support that *CESA6* plays major roles in cellulose deposition in roots under salt stress conditions, which is important for salt stress tolerance.

To check whether cellulose deposition defects affect salt stress gene expression, quantitative reverse transcription polymerase chain reaction was performed. Eight salt stress up-regulated genes were selected from the top list of a previous microarray experiment (Liu et al. 2007). It turned out that the degree of up-regulation of five genes by salt stress were much higher in roots of the *she1* (wt) plants than those that in the wild-type plants at least at one time point (Figure 1E). These results indicate that mutations in *CESA6* confer higher salt stress sensitivity and affect the expression of downstream stress responsive genes. Even though genes encoding proteins involved in carbohydrate transport and metabolism, lignification and formation of very long chain fatty acids were up-regulated by salt stress in *she1* roots, root growth of *she1* displayed a higher degree of inhibition by salt stress, probably due to its defect in cellulose synthesis.

Cellulose is synthesized by a large, plasma membrane-tethered cellulose synthase complex (CSC) that can be visualized as a hexameric rosette structure (Hill et al. 2014). There are 10 cellulose synthase (CESA) genes in the *Arabidopsis* genome (Richmond and Somerville 2000). CESA1, CESA3 and CESA6 are involved in primary cell wall synthesis, while CESA4, CESA7 and CESA8 are required for secondary cell wall synthesis (Endler and Persson 2011). Previously, it was reported that the *Arabidopsis cesa1*/*rsw1* mutant is salt sensitive; we found both *she1* and *prc1-1* roots are hyper-sensitive to salt stress, suggesting that CESA6 plays a similar role to CESA1/RSW1 (Kang et al. 2008) in *Arabidopsis* roots under salt stress conditions. Both *CESA1* and *CESA6* single mutants have a modest phenotype under normal growth conditions, which could be explained by the functional redundancy with other CESA genes. Salt stress may affect the activities of CESA and thus knocking out either CESA6 or CESA1 confers salt stress sensitivity. CESA6 interacts with cellulose synthase-interactive protein 1 (CSI1; Gu et al. 2010), a

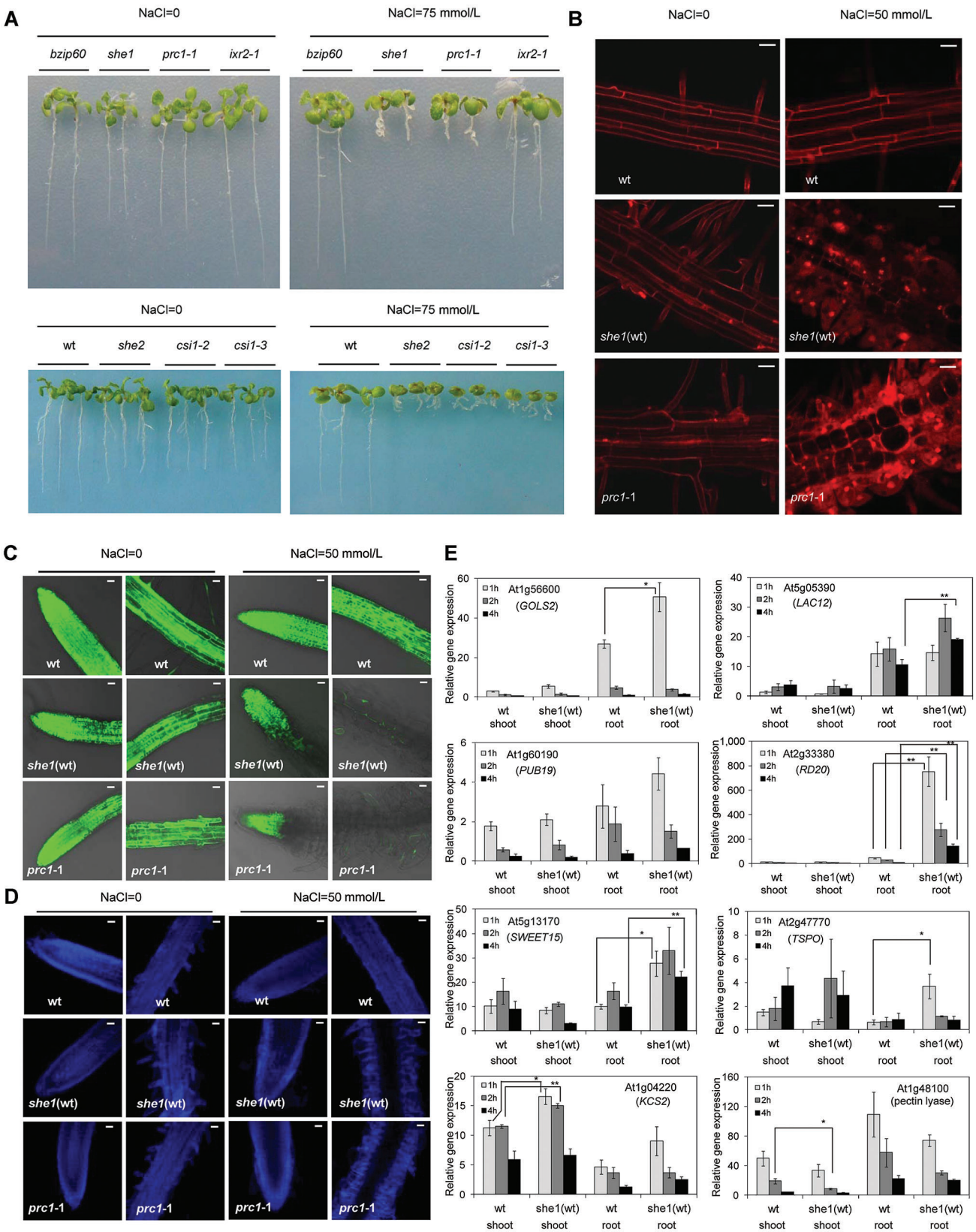

Figure 1. Continued.

large plant-specific protein that mediates the connection between CSC and cortical microtubules to guide cellulose synthesis at the plasma membrane (Li et al. 2012). Salt and osmotic stresses rapidly affect microtubule assembly (Komis et al. 2002), indicating that the CSC may be dynamically regulated in response to prolonged salt stresses in plants in which the CSI1 protein may have roles in reassembling the complex for cellulose synthesis. In our genetic screening, we isolated another *Arabidopsis* salt hyper-sensitive mutant, *she2* (Figures 1A, S6), in which *CSI1*/*POM2* was mutated (Figure S7; Table S2). *she2* and another two *csi1* mutant alleles (*csi1-2* and *csi1-3*) were all hyper-sensitive to salt stress (Figure 1A). Disruption of the link between CSC and cortical microtubules may affect the synthesis/deposition of cellulose at the plasma membrane under prolonged salt stress conditions. Taken together our results suggest that regulation of the cellulose synthase complex is very important for cellulose synthesis, therefore also for root growth under salt stress conditions.

Previously, a cellulose synthase-like protein, SOS6, was demonstrated to confer osmotic stress tolerance in *Arabidopsis* (Zhu et al. 2010). However, the *sos6-1* mutant has a modest defect in cell wall formation, and both shoots and roots of the *sos6-1* mutant are sensitive to salt-induced osmotic stress (Zhu et al. 2010). These contrasting results suggest that SHE1 and SHE2 play distinct roles from SOS6 in response to salt stress. Thus, our forward genetic analysis in *Arabidopsis* has revealed that sustained cellulose synthesis conferred by *CESA6* and *CSI1* is important for salt stress tolerance.

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Figure 1. Sustained cellulose synthesis is required for salt stress tolerance

(**A**) Phenotypes of *CESA6* and *CSI1* mutants. *CESA6* mutants (upper) and *CSI1* mutants (lower) were grown on 1/2 MS plates without or with low concentration of NaCl (75 mmol/L) for 10 d and then photographed. (**B, C**) Membrane integrity and cell viability of *CESA6* mutants. The wild-type (wt) and *CESA6* mutants were grown on 1/2 MS plates without or with low concentration of NaCl (50 mmol/L) for 14 d, and their roots were stained with propidium iodide (PI, **B**) or fluorescein diacetate (FDA, **C**) and observed under confocal microscopy. Bar = 10 μm. (**D**) Cellulose deposition in roots of *CESA6* mutants. The wild-type (wt) and *CESA6* mutants were grown on 1/2 MS plates without or with low concentration of NaCl (50 mmol/L) for 14 d, and their roots were stained with calcofluor and observed under confocal microscopy. Bar = 10 μm. (**E**) Up-regulation of salt stress responsive genes in *CESA6* mutant. The wt and *CESA6* mutant plants were treated with 100 mmol/L NaCl for a specified time, and their roots and shoots were separated for further gene expression analysis. Relative gene expression is defined as the gene expression level of plants treated with NaCl divided by that of non-treated plants, both of which were normalized to the expression of actin. Bars depict SE ($n = 3$). $^{**}P < 0.01$, $^{*}P \leq 0.05$. Comparisons with $P > 0.05$ are not noted. GOLS2, galactinol synthase 2; LAC12, laccase 12; PUB19, plant U-box protein 19; RD20, responsive to dehydration 20; SWEET15, sucrose efflux 15; TSPO, membrane-bound protein; KCS2, 3-ketoacyl-CoA synthase 2.

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S.S.Z. performed the research with the help of L.S., X.D. and S.J.L. W.T. supervised the bioinformatics analysis. S.S.Z. and J.X.L. designed the experiment, analyzed the data and wrote the manuscript.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Doc 1. Plant materials, growth conditions and phenotypic analysis

Table S1. Candidate genes for *SHE1*

Table S2. Candidate genes for *SHE2*

Table S3. Primers used in the current study

Figure S1. *she1* mutant is hyper-sensitive to salt stress

(**A, B**) Phenotypes of *she1* seedlings (**A**) or *she1* roots (**B**). Wild-type control (*bzip60*) and *she1* were grown on ½ MS plates without or with low concentration of NaCl (75 mmol/L) for 10 d and then photographed. Bar = 10 μm in **B**.

Figure S2. *she1* mutant is hyper-sensitive to both ionic and osmotic stresses

(**A-D**) Dose responses of wild-type control (*bzip60*) and *she1* seedlings to NaCl, KCl, LiCl and mannitol. Data are expressed as the relative root growth after 10 days in which root length on stress-imposed growth medium normalized to the root length on normal growth medium. Ten seedlings were scored in each of three replicates and bars depict SE ($n = 3$).

Figure S3. *SHE1* is isolated through deep-sequencing after rough mapping

Physical map of the region of chromosome 5 containing the *SHE1* gene. Rough mapping located *SHE1* in the region between MQB2 and MPA24. Direct deep-sequencing of *she1* mutant revealed a non-sense mutation in the *CESA6* locus (AT5G64740). Other two previously identified mutant alleles (*prc1-1* and *ixr2-1*) are also depicted. CESA6 contains 13 exons (boxes) and 12 introns (lines).

Figure S4. SHE1 encodes a cellulose synthase CESA6

(**A, B**) Allelic test between *she1* and *prc1-1*. F1 plants from across between *she1* and *prc1-1* were genotyped (**A**) and their sensitivity to salt stress was examined (**B**). *she1* is in the *bzip60* mutant background, therefore the heterozygosity of T-DNA was used for genotyping. (**C**) Independent of *she1* on *bZIP60* mutation. *she1* mutant was crossed to the wild-type and *SHE1* mutant in the wild-type (wt) background (*she1*(wt)) was identified in the F2 population by genotyping. The salt stress sensitivities of *bzip60*, *she1* and *she1* (wt) were compared when growing on ½ MS plates without or with low concentration of NaCl (75 mmol/L) for 10 d and then photographed.

Figure S5. Mutation of *CESA6* increases lateral root emergence under salt stress conditions which is related to auxin

(**A, B**) The wild-type (wt) and *she1* (wt) plants were grown on 1/2 MS plates for 4 d vertically, then transferred to 1/2 MS plates with or without low concertation of NaCl (75 mmol/L), or with NaCl (75 mmol/L) plus auxin efflux inhibitor NPA (0.5 μmol/L) for another 8 d and then photographed (**A**), and the number of their lateral roots was counted (**B**). Ten seedlings were scored in each of three replicates and bars depict SE ($n = 3$).

Figure S6. *she2* mutant is hyper-sensitive to salt stress

Wild-type control (*bzip60*) and *she2* were grown on 1/2 MS plates without or with low concentration of NaCl (75 mmol/L) for 10 d and then photographed.

Figure S7. SHE2 encodes a cellulose synthase-interactive protein CSI1

(**A**) *SHE2* is isolated through deep-sequencing after rough mapping. Physical map of the region of chromosome 2 containing the *SHE2* gene. Rough mapping located *SHE2* in the region between F3P11 and K1G2. Direct deep-sequencing of *she1* mutant revealed a non-sense mutation in the *CSI1* locus (AT2G22125). Anther two previously identified T-DNA mutant alleles (*csi1-2*, *SALK_122304*; *csi1-3*, *SALK_138584*) are also depicted. CSI1 contains 8 exons (boxes) and 7 introns (lines).

(**B, C**) Allelic test between *she2* and *csi1-2*. F1 plants from a cross between *she2* homozygous and *csi1-2* heterozygous were genotyped (**B**) and their sensitivity to salt stress was examined (**C**). *csi1-2* is a T-DNA mutant therefore the heterozygosity of T-DNA was used for genotyping.